

# The relationship between blood nitric oxide levels and brain infarct volume in patients with ischemic stroke

Comparison nitric oxide levels and ischemic volume in stroke

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## Abstract

**Aim:** Cerebrovascular disease (CVD) is the third leading cause of morbidity and mortality. Most risk factors disrupt the contraction and enlargement of the cerebrovascular endothelium. One of the main players in the maintenance of cerebrovascular homeostasis is nitric oxide (NO). In this study, we aimed to investigate the level of NO in patients with ischemic stroke and to determine the role of NO by comparing the infarct volumes of patients' brain as measured by computed tomography (CT) with the levels of NO.

**Material and Methods:** This is a prospective observational study. Blood samples and brain computed tomography images were taken at 0 and 24 hours from the ischemic stroke patients. Serum NO levels and the volumes of ischemic brain lesions were measured using CT imaging.

**Results:** In our study, we had 60 patients who are diagnosed with acute ischemic stroke and 37 healthy individuals. Mean NO levels in stroke patients were significantly lower compared with the control group. Patients were classified into 3 groups as 0-7, 8-14, 15≤ according to their NIHSS scores to compare their NO levels and infarct volumes. When the infarct volume of the group, which had an NIHSS score of 15≤ at 0 hours was compared with the other groups, it was found to be significantly higher ( $p<0.001$ ). NO levels in the same group at 0 hours were significantly lower than in the other groups.

**Discussion:** The relationship between serum NO levels and brain infarct volume in ischemic stroke patients was clearly demonstrated in our study.

## Keywords

Cerebrovascular Disease, Ischemic Stroke, Nitric Oxide, Infarct Volume

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## Introduction

Stroke is a clinical case occurring after sudden cerebral functional failure due to pathology of the vessels in the brain, and it is the third leading cause of death following cardiovascular diseases and cancer [1]. The most common type of stroke is ischemic stroke [2]. Ischemic stroke is caused by several risk factors such as hereditary predisposition, hypertension, diabetes mellitus, obesity, and elevated blood lipids, all of which might be independently associated with each other [3]. These risk factors may cause failure in cerebrovascular endothelin function, which plays a critical role in the regulation of cerebral blood flow [4]. Microvascular endothelial cells in the brain can produce and release many vasoactive substances, including nitric oxide (NO) and endothelin-1 that play a major role in the maintenance of cerebrovascular homeostasis [5,6].

NO is an inorganic gas that plays important roles in the control of cerebral blood flow, thrombogenesis, and the modulation of neuronal activity [7,8]. NO is produced in the endothelial cells, glial cells, neurons and macrophages by 3 different isoforms of the enzyme nitric oxide synthase (NOS) [9]. The identified functions of NO in the vascular system are regulation of vasomotor tonus, inhibition of thrombocyte adhesion and aggregation, suppression of cell proliferation and platelet activation, and the modulation of myocardial contraction [10]. Endothelial NO derived from endothelial nitric oxide synthase (eNOS) is the most important determinant of basal vascular tone, which regulates systemic circulation. NO is also involved in the regulation of local circulation of some organs (e.g., in the heart and brain) [11,12]. Studies have shown that both inactivation of eNOS genes by neural nitric oxide synthase (nNOS) inhibitors and eNOS deficiency cause hypertension in rats [11].

Risk factors predisposing to atherosclerosis are associated with a reduction in bioactive NO levels, and abnormal endothelial function. This depends on whether NO is truly deficient or inactivated by entering the reaction with oxygen-derived radicals. In addition, platelets also contribute to the control of platelet activation by synthesizing NO [13,14]. A growing number of studies were conducted on the relationship between stroke and NO levels in order to understand whether NO production and its levels are important in terms of the diagnosis, prognosis and prevention of cerebrovascular diseases. Therefore, this study aims to investigate the relationship between blood NO levels and infarct volumes of the brain measured by computed tomography in patients with ischemic stroke.

## Material and Methods

### Study design and patients

This is a prospective observational study conducted between November 2017 and January 2018 on ischemic stroke patients who were admitted to the emergency room and were diagnosed with ischemic stroke. We received ethical approval from Clinical Research Ethics Committee of Izmir Bozyaka Training and Research Hospital (No:8.11.2017/4). The study population consisted of 60 patients with a first episode of hemispheric ischemic stroke within 24-hour after the onset of symptoms. Thirty-seven healthy subjects served as controls. Systemic and neurological examinations were performed following a detailed anamnesis of each patient.

### Assessments and analyses

The severity of the neurological deficit was determined by the National Institute of Health Stroke Scale (NIHSS) score. The NIHSS is widely used to assess the severity of acute ischemic stroke. For a more detailed study of the patients, we divided them into 3 groups according to the NIHSS scale. Patients were grouped according to the NIHSS scoring system as mild (0-7), moderate (7-14), and severe (greater than 15, 15≤). Routine blood tests and ECG were also done. Cerebral infarct location was detected using CT. The aforementioned assessments and tests were performed again together with neurological examination 24 hours after the first admission. Venous blood samples (8-mL vacuum and gel separator tubes, BD Vacutainer SST™ II Advance) were collected and centrifuged for 10 minutes at 3,000 rpm after blood clotted. The sera were decanted and stored at -80 °C until the ELISA assay. Serum NO levels were determined using a commercial ELISA kit (Andy Gene Biotechnology, China) according to the manufacturer's instructions. CT was used to find and measure the ischemic area of the brain at the time of the first admission and 24 hours after the first admission. The volume of the ischemic area of the brain was calculated according to the Cavalieri method by multiplying the surface area of the lesion with the imaging cross-sectional thickness (5mm) (Figure 1) [15].

### Statistical analysis

All data were evaluated using the SPSS version 22.0 package program. Data were expressed as mean ± SD (Standard Deviation) values in the tables. The data were evaluated using Shapiro-Wilk for normal distribution. The Wilcoxon Sign Test was conducted for dependent variables and the Kruskal-Wallis Test was done for more than two independent groups when assumptions of normal distribution and/or homogeneity of variance were violated. The Mann-Whitney U test was performed between two independent groups that did not meet the normal distribution when necessary. In all patients, Spearman's correlation analysis was used to examine correlations between NO levels and infarct volumes. Since the data between the groups separated according to the NIHSS score were not normally distributed, Logarithmic transformation was performed followed by a one-way ANOVA test on these data. Duncan's Post-Hoc analysis was performed to determine which groups were different because the relationship between infarct volume and NO levels was significant among these groups. The Mann-Whitney U test was performed because the data were not normally distributed when comparing NO levels in the control group and the patient group at 0 hours. The effects of group and gender on NO levels in the NIHSS groups were evaluated by a two-way analysis of variance. Among the NIHSS score groups, a two-way ANOVA test was used for repeated measurements to compare NO levels at 0 and 24 hours. Statistical significance level was taken  $p < 0.05$  in all tests.

## Results

Baseline characteristics of patients with stroke are shown in Table 1; 84.4% of patients were diagnosed with at least one comorbid disease, while 16.6% of them had no comorbid disease. For control subjects (n=37), the mean age of males (n=20) and females (n=17) was  $51.08 \pm 8.37$  (Table 1).

Table 1. Baseline characteristics of individuals in the study

| Characteristics                     | Patients with stroke             | Control                         |
|-------------------------------------|----------------------------------|---------------------------------|
| Age (male/female)                   | 69.38 ± 12.48<br>(68.82 / 70.63) | 51.081 ± 8.367<br>(51.39/52.33) |
| Gender (male/female) (%)            | 33/27 (55%-45%)                  | 20/17<br>(54.1%-45.9%)          |
| Diabetes (no/yes) (%)               | 36/24 (40%)                      |                                 |
| Hypertension (no/yes) (%)           | 19/41 (68%)                      |                                 |
| Cardiovascular disease              | 45/15 (25%)                      |                                 |
| Coronary artery disease             | 44/16 (26.6%)                    |                                 |
| Hyperlipidemia                      | 38/22 (36.6%)                    |                                 |
| Echo (pathologic/normal)*           | 36/24 (60%)                      |                                 |
| Carotid artery stenosis or plaque   | 40/20 (66.6%)                    |                                 |
| Vertebral artery stenosis or plaque | 10/50 (16.6%)                    |                                 |
| ECG (normal / AF)                   | 48/12 (80%/20%)                  |                                 |

\* Pathologic findings are left ventricular hypertrophy, valve insufficiency, left atrial enlargement, and thrombus, ECG; Electrocardiogram, AF: Atrial fibrillation

Table 2. According to NIHSS scores, NO levels and brain infarct volumes at 0 and 24 hour

|                     | 0-hour NO levels μmol/L     | 24-hour NO levels μmol/L     | Brain infarct volumes (cm³)   |
|---------------------|-----------------------------|------------------------------|-------------------------------|
| 0-hour NIHSS score  |                             |                              |                               |
| 0-7 (n=32)          | 22.366 ± 6.857              | 29.924 ± 8.516               | 5.040 ± 7.373                 |
| 8-14 (n=20)         | 21.957 ± 7.0798             | 24.366 ± 8.777               | 17.726 ± 11.964               |
| 15≤ (n=8)           | 14.472 ± 2.984 <sup>a</sup> | 14.92 ± 3.464 <sup>a</sup>   | 69.032 ± 30.793 <sup>#</sup>  |
| 24-hour NIHSS score |                             |                              |                               |
| 0-7 (n=37)          | 22.539 ± 7.746              | 30.163 ± 8.447               | 5.224 ± 2.427                 |
| 8-14 (n=16)         | 18.401 ± 5.288              | 20.913 ± 6.384               | 23.110 ± 14.539               |
| 15≤ (n=7)           | 15.274 ± 3.585 <sup>b</sup> | 15.247 ± 3.585 <sup>**</sup> | 68.392 ± 31.103 <sup>##</sup> |

<sup>a</sup> p < 0.001 NIHSS 15 ≤ group when compared with NIHSS 0-7 and 8-14 groups (at 0 hours).  
<sup>b</sup> p < 0.001 NIHSS 15 ≤ group when compared with NIHSS 0-7 and 8-14 groups (at 24 hours).  
<sup>\*</sup> p < 0.001 NIHSS 15 ≤ group when compared with NIHSS 0-7 and 8-14 groups (at 0 hours).  
<sup>\*\*</sup> p < 0.001 NIHSS 15 ≤ group when compared with NIHSS 0-7 and 8-14 groups (at 24 hours).  
<sup>#</sup> p < 0.001 NIHSS 15 ≤ group when compared with NIHSS 0-7 and 8-14 groups (at 0 hours).  
<sup>##</sup> p < 0.001 NIHSS 15 ≤ group when compared with NIHSS 0-7 and 8-14 groups (at 24 hours).

Table 3. NO levels and brain infarct volumes of patients in decreased, increased and unchanged NIHSS score groups (at 24 hours)

|                          | Unchanged NIHSS score group (n=35) | Decreased NIHSS score group (n=18) | Increased NIHSS score group (n=7) |
|--------------------------|------------------------------------|------------------------------------|-----------------------------------|
| 0 hour NO levels μmol/L  | 19.29 ± 6.891                      | 22.429 ± 7.287                     | 24.485 ± 5.191                    |
| 24 hour NO levels μmol/L | 26.666 ± 9.067                     | 26.444 ± 10.851                    | 19.572 ± 7.158 <sup>ab</sup>      |
| Infarct volumes (cm³)    | 15.188 ± 23.056                    | 14.307 ± 16.740                    | 62.394 ± 32.9 <sup>*</sup>        |

<sup>\*</sup> p < 0.001 Increased NIHSS score group when compared with the decreased and unchanged NIHSS score groups.  
<sup>a</sup> p > 0.05 Increased NIHSS score group when compared with the decreased and unchanged NIHSS score groups.  
<sup>b</sup> p > 0.05 NO levels of increased NIHSS score group (at 24 hours) when compared with the NO levels of increased NIHSS score group (at 0 hours).

The mean NO level in the control group was 37.965 ± 7.269 μmol/L (male / female 39.315 ± 7.223 / 36.376 ± 6.824 μmol/L). In the patient group, it was 21.184 ± 7.2847 at 0 hours and 25.955 ± 9.372 μmol/L at 24 hours. According to gender, the male/female ratio at 0 hours was 21.56 ± 6.860/20.715 ± 7.646 μmol/L. At the 24th hour, it was 26.16 ± 8.814/25.701 ± 9.830 μmol/L. We found that gender had no effect on NO levels (p>0.05). The mean NO levels for the entire patient group at 0 and 24 hours were significantly lower when compared to the control group (p<0.001). We also found that the mean NO level

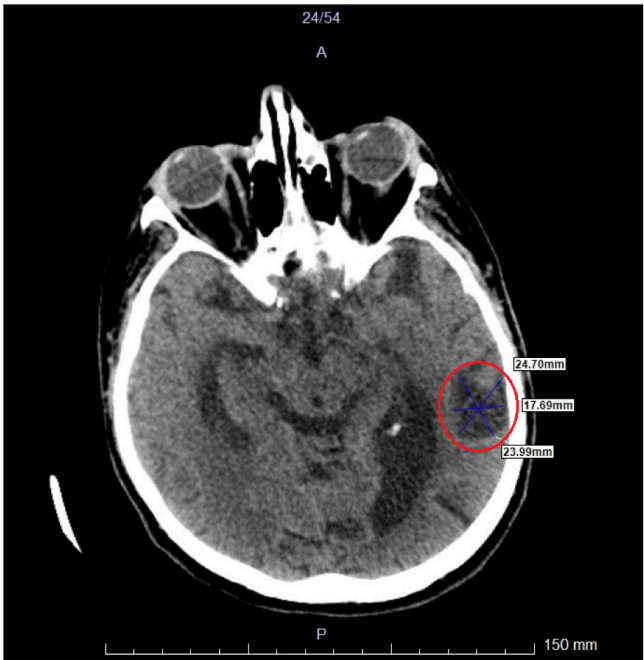


Figure 1. Cavalieri method

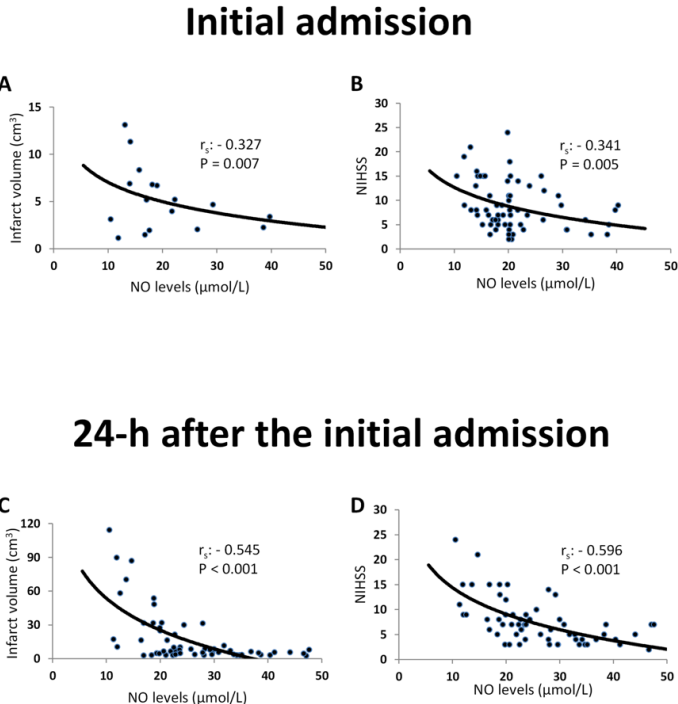


Figure 2. Correlation analysis of altered NIHSS scores, infarct volumes and NO levels at 0 hours and 24 hours

in the patient group at 24 hours increased compared to the 0- hour level. The mean NIHSS score of the patients at the time of admission (0 hours) was calculated as 8.016 ± 4.335. NIHSS score was 0-7 in 33 patients (53.3%), NIHSS score was 8-14 in 19 patients (33.3%) and NIHSS score was 15 and over in 8 patients (13.3%). Additionally, the mean NIHSS score of the patients at 24 hours was calculated as 7.6 ± 4.603. NIHSS score at 24 hours was between 0-7 in 37 patients (61.6%), between 8-14 in 16 patients (26.6%), and 15 and over in 7 patients (11.6%). When the patients were grouped according to their NIHSS scores at 0 hours, mean NO levels of the patients with NIHSS score 15 and over were significantly lower than NO levels in the

other score groups ( $p < 0.001$ ), although a significant increase was observed in infarct volume of patients NIHSS score  $15 \leq$  ( $p < 0.001$ ). Likewise, mean NO levels of the patients with NIHSS score of  $15 \leq$  at 24 hours were significantly lower than in other groups at 24 hours ( $p < 0.001$ ). In the same manner, there was a significant increase in infarct volume of patients' NIHSS score  $15 \leq$  at 24 hour ( $p < 0.001$ ) (Table 2).

Patients were divided into 3 groups according to their altered NIHSS scores when comparing the difference between 0 and 24 hours, as increased NIHSS score, decreased NIHSS score, and unchanged NIHSS score. NIHSS scores were increased in 7 patients, decreased in 18 of them, and no changes were found in 35 of them. It was found that the NO levels measured at 24 hours in patients with increased NIHSS score group were not statistically significant, but were lower than the NO levels at 0 hours ( $p > 0.05$ ). In addition, it was observed that the mean infarct volume was significantly higher in the increased NIHSS score group compared to the other groups ( $p < 0.001$ ). Likewise, in the group with increased NIHSS score, it was observed that NO levels at 24 hours were lower than the 0-hour levels, although it is not statistically significant, while the opposite situation was also observed in the group with a decreased NIHSS score (Table 3).

Regarding the results of comparison between altered NIHSS score groups in Table 3, we further analyzed the relationship among these groups, and used Spearman's correlation analysis for this purpose. We found statistically significant negative correlations between both infarct volume-NO levels and NIHSS scores-NO levels at 0 and 24 hours ( $p < 0.01$  and  $p < 0.001$ ) (Figure 2).

## Discussion

Studies have shown that early diagnosis and treatment of patients who come to emergency services with CVD can reduce the effects of this disease on mortality and morbidity. Thus, for use in the early diagnosis and treatment of patients with ischemic stroke, studies with many molecules that are effective in pathogenesis are being made. Chemerin, basic fibroblast growth factor (bFGF), Adropine, Pentraxin 3 and NO are some of them [14,16].

One of the most important factors affecting the pathogenesis of ischemic stroke is the deterioration of endothelial function. In patients exposed to vascular risk factors, endothelium-dependent relaxation dysfunction is detected prior to morphological changes in the cell wall [17,18]. According to a study, such as hypertension, hyperlipidemia, smoking and diabetes, abnormal endothelial functions have been shown to be associated with a decrease in NO levels [19].

NO is a potent vasodilator synthesized by the nitric oxide synthase enzyme from L-arginine. NO inhibits platelet aggregation, leukocyte chemotaxis and their adhesion, as well as downregulates chemokine expression, reduces smooth muscle cell proliferation, migration and LDL oxidation, and thus it has anti-atherogenic and anti-thromboembolic effects [11,19]. In studies conducted by Drake and colleagues, it has been shown that the most important signaling molecule that plays a role in the autoregulation of cerebral blood flow and in cerebrovascular endothelium is endothelial NO [11].

In general, according to some studies, loss of endothelial NO is considered to be the central mechanism in the pathogenesis of endothelin dysfunction [19,20]. In both cerebral and peripheral vasculature, a decrease in endothelial NO results in impaired vascular function and processes such as vasoconstriction, increase in arterial blood pressure, proliferation of vascular smooth muscle cells, platelet aggregation, adhesion of white blood cells, and inflammation. For this reason, it has been found that the preservation of endothelial NO production is important for the prevention of cerebrovascular diseases [14].

Many animal experiments have been carried out in order to understand the mechanism of NO action at the molecular level. In animal studies performed, the infarct area due to middle cerebral artery occlusion was found to be larger in the eNOS Knock-out mice than the control group. Activation of eNOS has been shown to provide protection against stroke by preventing inflammation, platelet aggregation, thrombosis, and apoptosis. According to these studies, it has been demonstrated that NO is an important mediator in the regulation of cerebral blood flow [10,21].

In a study by Rashid and colleagues, plasma levels of NO in 38 controls, 228 ischemic stroke, and 49 hemorrhagic stroke patients were examined, and as a result, levels of NO in control group was  $64.0 \pm 36.3 \mu\text{mol/L}$ ,  $49.9 \pm 26.1 \mu\text{mol/L}$  in the group of ischemic stroke and  $41.7 \pm 19.5 \mu\text{mol/L}$  in the group of hemorrhagic stroke [22]. Nandhagopal found that serum NO levels were lower in stroke patients ( $40.9 \pm 3.9 \mu\text{mol/L}$ ) than in the control group ( $59.9 \pm 7.3 \mu\text{mol/L}$ ) in a study of 40 stroke patients (Nandhagopal R, Krishnamoorhy SG, Vengamma B. Circulating Levels of Nitric Oxide in Stroke, Poster Abstracts, Sri Venkateswara Institute of Medical Sciences. 2005, Tirupati, India). In another study, the NO levels in 81 patients with acute ischemic stroke were  $58.46 \pm 1.92 \mu\text{mol}$  and were  $61.22 \pm 0.95 \mu\text{mol}$  in 50 healthy controls (Bengü Ş. Evaluation of asymmetric dimethylarginine, endothelial nitric oxide synthase and nitric oxide as a risk factor for early-onset ischemic stroke cases in Eastern Anatolia Region, (PhD Thesis), Erzurum, 2014.). In contrast to all of these data, Moro et al. found that NO levels in cerebrospinal fluid and in serum were higher in stroke patients than in the control group [23].

In our study, similar to other studies in the literature, mean NO levels in stroke patients were significantly lower ( $p < 0.001$ ). In addition, although the NO levels of patients measured at 24 hours were significantly increased compared to the 0-hour level, they were significantly lower than in the control group ( $p < 0.001$ ).

The infarct volumes were significantly higher in the group of NIHSS  $15 \leq$  at 0 hours when compared with the other groups ( $p < 0.001$ ). NO levels at 0 hours were significantly lower in the group with NIHSS score 15 and over compared with the groups of NIHSS score 0-7 and 8-14 ( $p < 0.001$ ).

According to the investigation among these groups, NO levels at 0 hours were significantly lower in the group of NIHSS score 15 and over when compared with the other groups, additionally, NO levels for the same group at the 24 hours were also lower ( $p < 0.001$ ). When we compared NO levels at 0 hours and 24 hours, the increase in NO levels for the NIHSS score 0-7 and 8-14 groups was significant ( $p < 0.001$ ). Nevertheless, there was

no change in the levels of NO in the group of NIHSS score 15 and over. According to these results, it can be said that the infarct volume may be lowered due to the protective effect of NO that occurs as a result of its increase.

To examine our data from another perspective, patients were divided into 3 groups according to the NIHSS score at 24 hours as increased, decreased and unchanged. In patients with an increased NIHSS score, NO levels decreased at 24 hours compared to 0 hours, and the infarct volumes were significantly higher than in the other groups ( $p < 0.001$ ). NO levels of the patients in the groups of decreased and unchanged NIHSS score at 24 hours were increased compared to 0 hours. These results also support the association between NO levels and infarct volume.

### Conclusion

Our study investigates the relationship between NO levels and infarct volume in stroke patients. In many experimental animal studies, NO activity and eNOS are associated with such disease models and their protective effects are known. This study highlights the protective effects of NO in ischemic stroke through demonstrating the significant relationship between infarct volumes and NO. However, the molecular mechanisms underlying this relationship need to be studied with more detailed animal studies, and should be strengthened by clinical studies involving even more patients.

### Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

### Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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### Conflict of interest

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